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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Sung Youb JUNG *et al.*
Application No.	:	10/535,312
Filed	:	June 5, 2006
Confirmation No.	:	5682
For	:	METHOD FOR THE MASS PRODUCTION OF IMMUNOGLOBULIN CONSTANT REGION

Examiner	:	Lynn Anne Bristol
Art Unit	:	1643
Docket No.	:	430156.404USPC
Date	:	February 27, 2009

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF SUNG YOUB JUNG AND JIN SUN KIM
UNDER 37 C.F.R. § 1.132

Dear Commissioner:

We, Sung Youb JUNG and Jin Sun KIM, hereby declare that:

1. Sung Youb Jung is a co-inventor of the above-referenced patent application ("the subject application") and is a Researcher, Team Leader in Research Laboratories, Hanmi Research Center, Hanmi Pharm. Co., Ltd, the assignee of the present invention; and Jin Sun KIM is a co-inventor of the subject application and is a Researcher in Hanmi Research Center, Hanmi Pharm. Co., Ltd, the assignee of the present invention.

2. We have reviewed the Office Action dated August 27, 2008, in the subject application, including the rejection under 35 U.S.C. § 112, first paragraph, for alleged lack of enablement, and submit this Declaration to provide evidence to the Examiner in support of the patentability of the presently claimed invention, which is directed generally to methods for the mass production of Immunoglobulin constant regions (hereinafter "Ig Fc region") in bacteria.

3. The Examiner has alleged that the specification of the subject application is not enabling for producing constructs comprising a heavy chain region that comprises a C_H1, hinge, C_H2, C_H3 (or C_H4) domains from any combination of different Ig molecules, among other combinations or hybrids of Ig constant regions.

4. We carried out studies in accordance with the teachings of the subject application to further illustrate that hybrids of Ig constant regions comprising one to four domains selected from the group consisting of C_H1, C_H2, C_H3, and C_H4 could be generated using nothing more than routine experimentation.

5. The procedure we carried out is as follows:

A. Cloning of STII-G2-4 hybrid. To minimize the effector function of the immunoglobulin fragment, the hybrid Fc of IgG2 and IgG4 was generated from the single hybrid gene which was cloned by using the sequence of IgG2 region (hinge region and parts of CH2), known to have no ADCC (Antibody-dependent cell-mediated cytotoxicity) function, and the sequence of IgG4 (CH2 and CH3), known to have no CDC (complement-dependent cytotoxicity) function.

To construct the STII-IgG2-4 hybrid clone, the G2-4 hybrid gene (which is the insert sequence to the cloning vector) was obtained from the plasmid pSTIIdCG4Fc as a template by the method of degenerate PCR. The sense strands primer used in PCR had a G2 hinge sequence of 9 bases at the 5' region, and in the pSTIIdCG4Fc template binding region, the IgG2 CH2 codon was inserted (*see* sequences below). To facilitate the gene cloning, a BamHI restriction site was

added to the anti-sense primer. The amplified gene fragment of G2-4 was separated as a fragment of the size 600~700bp, through 0.8% agarose gel electrophoresis.

The primer sequences are as follows:

G2CGinfSS: CCGTGCCCAGCACCTCCGGTGGCAGGACCGTCA

G4BHIAS: CGCGGATTCTCATTTACCCAGAGACAGGGAGAGG

The cloning was performed using the plasmid pmSTII vector (wherein the site-directed mutagenesis was performed at the sequence coding the last amino acid residue of E.coli heat-stable enterotoxin signal sequence to change it to StuI restriction site). The pmSTII vector was treated with StuI and BamHI, and was then subjected to 0.8% agarose gel electrophoresis. A large fragment (4.7 kb) was obtained which contained the E. coli heat-stable enterotoxin signal sequence derivative.

The above isolated IgG2-4 Fc gene fragment was digested with BamHI and ligated with the linearized expression vector, thus providing pSTIIG2-4Fc.

B) Expression of STII-G2-4 hybrid clone in 5L jar scale. *E.coli* BL21DE3 was transformed with the pSTII-G2-4Fc expression vector. A single colony of transformed bacteria was inoculated in 5 ml of LB medium, cultured for 12 hr, followed by preparation of a glycerol stock. The recombinant was then inoculated and cultured overnight in 200 ml of LB medium with agitation. This 200 ml culture was used as a seed culture for expression. 200 ml of the seed culture was inoculated in 2L LM media in a 5L jar fermentor (Marubishi), and cultured at 32°C, an air rate of 1vvm, and 500 rpm agitation. To increase the cell density and the protein expression level, the substrate was fed accordingly with the cell growth rate as the fermentation proceeded.

The substrate selected for the STII-G2-4Fc fermentation system was the mixture of glucose and yeast extract. The feeding rate of substrate was regulated according to the cell growth rate and pH change.. The amount of substrate was regulated to minimize the production of inhibiting by-product which inhibits the cell growth and the protein expression.

Protein expression was induced by the addition of IPTG at the OD value over 60 in 600 nm of absorbance. The culture was further incubated for 40 to 45 hrs and grown until the OD value at 600 nm reached to 120 to 140. The fermentation fluid was centrifuged (7,000 rpm, 20 minutes) to obtain the pellet, after which the supernatant was discarded. The pellet was used for the determination of protein expression. Osmotic shock was performed to determine protein expression. Sonication and western blotting were performed to determine the solubility of the protein. Protein A assay was carried out for identifying protein expression as a soluble form.

6. The results that we obtained are as follows:

A) Cloning of STII-G2-4 hybrid. Many possible candidate clones were obtained. The cloning was confirmed by enzyme analysis and by the sequencing. As a result, two clones having exact gene sequences were identified, and designated as "pSTII-G2-4Fc." To see the expression pattern, one of two clones was transformed into *E.coli* BL21DE3 strain. Confirming the presence of a hybrid of Ig constant regions, one clone, pSTII-G2-4Fc, included gene sequences of the following:

Sequence of STII

atg aaa aag aca atc gca ttt ctt ctt gca tct atg ttc gtt ttt tct att gct aca aat gcc cag gcg

Sequences of G2 hinge and parts of CH2

5' ccg tgc cca gca cct ccg gtg gca gga ccg tca gtc

Sequences of G4 Ch2~3

ttcctgttcccccaaaacccaaggacactctcatgatctcccggacccctgaggtcacgtgcgtggtggtggacgtg
agccaggaagaccccgaggtccagttcaactggtacgtggatggcgtggaggtgcataatgccaagacaaagccgc
gggaggagcagttcaacagcacgtaccgtgtggtcagcgtcctcaccgtcctgcaccaggactggctgaacggcaa
ggagtacaagtgcaaggtctccaacaaaggcctcccgtcctccatcgagaaaaccatctccaaagccaaagggcag
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accacgcctcccgtgctggactccgacggctccttcttcctctacagcaggctaaccgtggacaagagcaggtggca

ggaggggaatgtcttctcatgctccgtgatgcatgaggctctgcacaaccactacacacagaagagcctctccctgtct ctgggtaaatga 3'

B) Expression of STII-G2-4 hybrid clone in 5L jar scale. The condition for high density culture of the recombinant G2-4Fc strain in 5 L scale was confirmed as described above, and protein expression was determined by the following procedure. To determine whether expressed proteins were secreted into the periplasmic space of *E.coli* with the help of the signal sequence fused to the protein, the fermentation fluid was centrifuged and collected cells were subject to the osmotic shock. When the expression of protein in the periplasmic space was determined by SDS-PAGE and Western blot analysis, the amount was very small. The fermentation fluid was disrupted by ultrasonication (Misonix company), and the cell lysate was obtained. The resulting cell lysate was centrifuged to separate water-soluble substances from water-insoluble substances, and the substances were subjected to Western blot analysis. It was confirmed that most of the hybrid proteins were expressed as soluble substances. The level of protein expression (5.3 mg/L) was determined using the protein-A affinity column.

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information or belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the captioned patent application or any patent issued therefrom.

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Date Sung Youb JUNG

_____ _____

Date Jin Sun KIM

WTC:jto

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